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Hyperbaric oxygen downregulates ICAM-1 expression induced by hypoxia and hypoglycemia: the role of NOS

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[1]*Department of Emergency Medicine and* [2]*Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Brigham and Women's Hospital, Boston 02115;* [4]*Department of Pathology, Boston University School of Medicine, Boston, Massachusetts 02118; and* [3]*Department of Biomedical Sciences, Baylor College of Dentistry, Dallas, Texas 75246*

Buras, Jon A., Gregory L. Stahl, Kathy K. H. Svoboda, and Wende R. Reenstra. Hyperbaric oxygen downregulates ICAM-1 expression induced by hypoxia and hypoglycemia: the role of NOS. *Am. J. Physiol. Cell Physiol.* 278: C292–C302, 2000.—Hyperbaric oxygen (HBO) is being studied as a therapeutic intervention for ischemia/reperfusion (I/R) injury. We have developed an in vitro endothelial cell model of I/R injury to study the impact of HBO on the expression of intercellular adhesion molecule-1 (ICAM-1) and polymorphonuclear leukocyte (PMN) adhesion. Human umbilical vein endothelial cell (HUVEC) and bovine aortic endothelial cell (BAEC) induction of ICAM-1 required simultaneous exposure to both hypoxia and hypoglycemia as determined by confocal laser scanning microscopy, ELISA, and Western blot. HBO treatment reduced the expression of ICAM-1 to control levels. Adhesion of PMNs to BAECs was increased following hypoxia/hypoglycemia exposure (3.4-fold, $P < 0.01$) and was reduced to control levels with exposure to HBO ($P = 0.67$). Exposure of HUVECs and BAECs to HBO induced the synthesis of endothelial cell nitric oxide synthase (eNOS). The NOS inhibitor nitro-L-arginine methyl ester attenuated HBO-mediated inhibition of ICAM-1 expression. Our findings suggest that the beneficial effects of HBO in treating I/R injury may be mediated in part by inhibition of ICAM-1 expression through the induction of eNOS.

cell adhesion molecules; ischemia; reperfusion injury; hyperoxia; neutrophils

ISCHEMIA/REPERFUSION (I/R) injury represents a final common pathway in many disease states including myocardial infarction, stroke, compartment syndrome, and acute peripheral extremity ischemia (4, 12). Reperfusion injury is mediated in part through the effects of neutrophil infiltration into damaged tissue and free radical production (12, 19). Intercellular adhesion molecule-1 (ICAM-1) has been implicated in I/R injury by recruiting CD11/CD18-expressing polymorphonuclear leukocytes (PMNs) (23, 38). Inhibition of ICAM-1 function through blocking peptides, monoclonal antibodies, and an ICAM-1-null transgenic mouse model has shown a significant reduction in reperfusion injury (8, 17, 30, 39, 52). Nitric oxide (NO) also plays a role in I/R injury (reviewed in Ref. 22). Inhibition of endothelial cell-derived NO synthesis promotes the expression of ICAM-1 and the adhesion of PMNs in several models (10, 20). Also, infusion of organic NO donors decreases adhesion of PMNs to damaged endothelium (4a, 37).

Hyperbaric oxygen (HBO), exposure to oxygen at a pressure greater than one atmosphere absolute (ATA), has been used as an adjunctive therapy for several I/R injuries, including acute peripheral ischemia and myocardial infarction (4). Given the general association of high P_{O_2} with formation of reactive oxygen species (ROS) during reperfusion, the use of HBO in treating I/R injury appears counterintuitive. However, recent in vivo studies have shown that HBO treatment improves outcome following experimental I/R injury. A rodent raised pedicle muscle flap model demonstrated improved survivability of grafts following I/R with HBO treatment (41). Studies to date suggest that HBO may interfere with the destructive PMN infiltration response following I/R. Real-time videomicroscopy of HBO-treated muscle flaps following I/R have demonstrated decreased PMN adhesion to the endothelium and greater microvessel diameter (50). Also, absolute PMN content of HBO-treated muscle flaps following I/R injury is decreased vs. nontreated flaps (51). HBO pretreatment has been shown to decrease leukocyte adhesion and improve postischemic microvascular flow velocity and tissue ATP levels following I/R in a rodent liver model (5).

The molecular mechanisms responsible for the beneficial effect of HBO on PMN adhesion and infiltration are not well defined. This study sought to determine whether HBO affects the adhesion of PMNs to endothelium through modulation of ICAM-1 protein expression. We found that endothelial cell expression of ICAM-1 in vitro required exposure to both hypoxia and hypoglycemia. HBO treatment of endothelial cells following hypoxia/hypoglycemia stimulation abolished the induction of ICAM-1 protein expression and also the adhesion of PMNs to endothelial monolayers. HBO exposure also induced the production of endothelial nitric oxide synthase (eNOS). Inhibition of eNOS interfered with the HBO-mediated downregulation of ICAM-1 in our model. Our findings suggest that the beneficial effect of HBO in treating I/R injury may be mediated through decreased expression of ICAM-1,

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which in part may be related to an upregulation of eNOS activity.

MATERIALS AND METHODS

Reagents. Lipopolysaccharide (LPS), anti-β-tubulin antibody, and nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The MY-13 monoclonal anti-ICAM-1 antibody was purchased from Zymed Laboratories (CA). Anti-eNOS and anti-inducible NO synthase (anti-iNOS) primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Transduction Laboratories (Lexington, KY).

Cell culture and in vitro model of I/R. Briefly, early passage primary bovine aortic endothelial cells (BAECs; a kind gift from Dr. D. Larson, Boston University), human umbilical vein endothelial cells (HUVECs), or the RAW 264.7 murine macrophage cell line (generously provided by Dr. M. J. Fenton, Boston University) were plated in 60-mm tissue culture dishes or Labtek 4 chamber slides (Nunc, Marsh Biomedical, NY). Cells were grown in low-glucose (100 mg/dl) DMEM (GIBCO BRL), supplemented with 10% fetal bovine serum (Hyclone). To simulate ischemia in vitro, cells were exposed to hypoxia and hypoglycemia (28). Culture media were replaced with DMEM (containing 0 mg/dl glucose) and cells were placed in a modular incubator chamber (MIC-101, Billups-Rothenberg, Del Mar, CA). Room air gas, P_{O_2} ~150 mmHg (normoxic), in the incubator chamber was then exchanged with gas containing 95% N_2 and 5% CO_2 (hypoxic) by equilibration flow for 30 min. These conditions acutely reduce culture media P_{O_2} content to ~25–29 mmHg (28). Cells were incubated under mock ischemic conditions for 4 h, a time period chosen to mimic prior in vivo pedicle flap I/R models (50). After 4 h of mock ischemia, mock reperfusion was simulated by changing culture medium to low-glucose DMEM (normoglycemic) and incubating cells under normoxic conditions for an additional 20 h, for a total time of 24 h. In experiments utilizing L-NAME, this compound was diluted to final concentration and added to the cells at the time corresponding to the beginning of the reperfusion period.

HBO exposure. Experimental treatment with 100% O_2 at 1.0, 1.5, 2.0, or 2.5 ATA for 90 min was performed on initiation of the reperfusion phase after replacing hypoglycemic media with low-glucose DMEM. Access to a Sechrist experimental hyperbaric animal research chamber 1300B was kindly provided by Dr. R. Fabian, Massachusetts Eye and Ear Infirmary. HBO treatment conditions were selected to mimic in vivo models and current human treatment protocols for I/R injury (2, 50). After HBO treatment, cells were incubated under normoxic conditions for 18.5 h (total reperfusion period 20 h) unless otherwise indicated. Cells from all experimental groups were >95% viable throughout the studies as determined by trypan blue exclusion.

Confocal laser scanning microscopy. Membrane expression of ICAM-1 protein was analyzed on nonpermeabilized cells with a Leica confocal laser scanning microscope using a monoclonal ICAM-1 antibody (14). Briefly, cells were washed with 1× PBS and fixed with 4% paraformaldehyde. Cells were incubated with a 1:1,000 dilution of a monoclonal anti-ICAM-1 antibody recognizing the extracellular portion (Clone MY-13, Zymed) for 2 h at 25°C. Cells were then rinsed with 1× PBS and blocked for 30 min with goat serum, washed with 1× PBS, incubated with a secondary FITC-conjugated antibody, and rinsed and mounted. Measurement of intracellular eNOS or iNOS was performed by membrane permeabilization with 0.01% Triton X-100 for 5 min after initial fixation. Cells were washed with 1× PBS four times and processed as above, except that anti-eNOS or anti-iNOS antibodies were used primarily at a 1:1,000 dilution. Negative controls included cells incubated with secondary antibody alone and irrelevant mouse IgG primary antibody. Propidium iodide staining of nucleic acid was performed using a concentration of 2 μg/ml for 10 min before final washes. Confocal laser scanning microscopy (CLSM) quantitation was determined by analysis of fluorescence intensity. For quantitation of fluorescence intensity, the background laser intensity was set to untreated control conditions, and all subsequent samples were scanned under these conditions so that increases in ICAM-1 signal intensity relative to controls could be determined. Fluorescence intensity was measured in 12–20 representative cells from different slides for each condition by a blinded operator and statistically analyzed as described below. Each experiment was repeated a minimum of three times.

Cell surface immunoassay. ICAM-1 cell surface protein was quantified by ELISA as previously described with the following minor modifications (54). Briefly, cells were plated at a density of 10^5 cells per well in 96-well flat-bottomed culture plates and exposed to experimental conditions. For ELISA, cells were washed four times with Hanks' balanced salt solution (HBSS) and incubated with a 1:1,000 dilution of the MY-13 monoclonal anti-ICAM-1 antibody for 30 min at 37°C. Cells were then washed four times with HBSS and incubated with a 1:2,000 dilution of HRP-conjugated secondary IgG for 1 h. Cells were washed four times with HBSS, and a developing solution containing 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), final concentration 1 mM, was added for 10 min. Sample optical density was analyzed using a microtiter plate spectrophotometer (Molecular Devices) at 405 nm. Conditions were replicated with 6–24 wells per condition in each experiment, and each experiment was repeated a minimum of three times.

Western blotting. Cells were analyzed for total ICAM-1 and eNOS protein by Western blot analysis as follows. Briefly, total protein was extracted in buffer containing 50 mM Tris·HCl (pH 8.0), 0.15 M NaCl, 0.5% deoxycholate, and 1% Triton X-100 in the presence of 1 μg/ml aprotinin and 75 μg/ml phenylmethylsulfonyl fluoride. Samples were sonicated for 1–3 s, and insoluble material was removed by centrifugation. Twenty micrograms of each protein sample was separated on 7.5% SDS-PAGE and transferred to nitrocellulose paper at 70 V in a Trans-Blot electrophoretic transfer cell (Bio-Rad, Richmond, CA) while being cooled to 4°C on an Iso-Temp refrigerated circulator (Fisher Scientific). Nitrocellulose blots were blocked for 1 h in 1× PBS containing 5% milk and rinsed three times for 10 min with 1× PBS. Blots were then incubated with a primary anti-ICAM-1 monoclonal antibody (Zymed), anti-eNOS (Transduction Laboratories), or β-tubulin (Sigma) at a 1:1,000 dilution at 4°C for 12 h. Nitrocellulose blots were rinsed three times for 10 min in PBS at 4°C and incubated with HRP-conjugated goat anti-mouse IgG secondary antibody at 1 μg/ml final concentration for 1 h. Blots were rinsed three times in 1× PBS and incubated overnight at 4°C with 1× PBS containing 2% milk. Protein was detected using the enhanced chemiluminescence Western blotting detection system (Amersham, Arlington Heights, IL). Blots were wrapped in plastic wrap and exposed to autoradiograph film for various times. Signal intensity corresponding to ICAM-1 expression was quantified by scanning densitometry using National Institutes of Health (NIH) Image 1.5 software (NIH, Bethesda, MD). Changes in ICAM protein expression are expressed relative to baseline control protein expression.

PMN/endothelial cell adhesion assay. PMN isolation and adhesion to confluent endothelial cell monolayers was carried out as previously described (6, 54). Adhesion of human PMN to BAECs has been previously described (11). Briefly, BAECs were cultured in flat-bottomed 96-well plates at a density of 10^5 cells/well and exposed to experimental conditions. Plates were washed four times with HBSS, and 2.5×10^5 PMNs were added to each well and incubated at 37°C for 30 min. Quantitaion of bound PMNs was determined by measuring total myeloperoxidase activity by functional assay. Developing solution containing 1 mM 2,2′-azino-di-3-ethyl dithiazoline sulfonic acid with 10 mM H_2O_2 and 0.5% Triton X-100 in 100 mM citrate buffer (pH 4.2) was added to facilitate cell lysis and provide substrate for assay of myeloperoxidase enzymatic activity. Plates were incubated at 37°C for 10 min, and sample optical density was analyzed using a microtiter plate spectrophotometer (Molecular Devices) at 405 nm. Conditions were replicated with 3–6 individual samples per experiment, and each experiment was repeated a minimum of three times.

Statistical analysis. Statistical analysis was performed by ANOVA with Fisher's protected least-significant differences test using the statistical analysis software package Statview (Abbacus, Berkeley, CA).

RESULTS

Both hypoxia and hypoglycemia are required to induce ICAM-1 protein expression in endothelial cells: hypoxia/hypoglycemia-induced ICAM-1 is downregulated by HBO exposure. Previous studies have suggested that ICAM-1 protein expression is not induced by hypoxia alone and requires other stimuli such as LPS, cytokines, or a reperfusion period (13, 29, 49, 53). We sought to determine whether hypoglycemia was capable of inducing ICAM-1 alone or modifying ICAM-1 production in combination with hypoxia/reoxygenation. HUVECs were exposed to 4 h of hypoxia, hypoglycemia, or hypoxia and hypoglycemia, followed by 20 h of normoxia and normoglycemia. CLSM analysis was used to determine the induction of ICAM-1. CLSM demonstrates low levels of ICAM-1 protein in untreated control HUVECs (Fig. 1*A*). Four hours of hypoxia or hypoglycemia alone failed to induce ICAM-1 at 24 h (Fig. 1, compare *A*, *C*, and *D*). The combination of 4 h of hypoxia and hypoglycemia was sufficient to induce ICAM-1 protein expression at 24 h (Fig. 1, compare *A* and *E*). ICAM-1 expression following hypoxia/hypoglycemia was similar to levels following LPS stimulation (Fig. 1, compare *B* and *E*).

Having defined conditions in vitro leading to the induction of ICAM-1, we sought to determine whether HBO treatment of endothelial cells could inhibit ICAM-1 protein induction. The mock I/R protocol was modified to include 90 min of HBO at 2.5 ATA following the hypoxic/hypoglycemic period and preceding the normoxic/normoglycemic period. HUVECs subjected to hypoxia/hypoglycemia alone were transferred directly into normoxic/normoglycemic conditions for an equivalent 90-min period. After a total time of 24 h, cells were harvested and analyzed for ICAM-1 protein expression by CLSM. HBO treatment of hypoxia/hypoglycemia-treated endothelial cells reduced expression of ICAM-1 protein to control levels (Fig. 1, compare *A*, *E*, and *F*). HBO exposure alone did not affect ICAM-1 expression

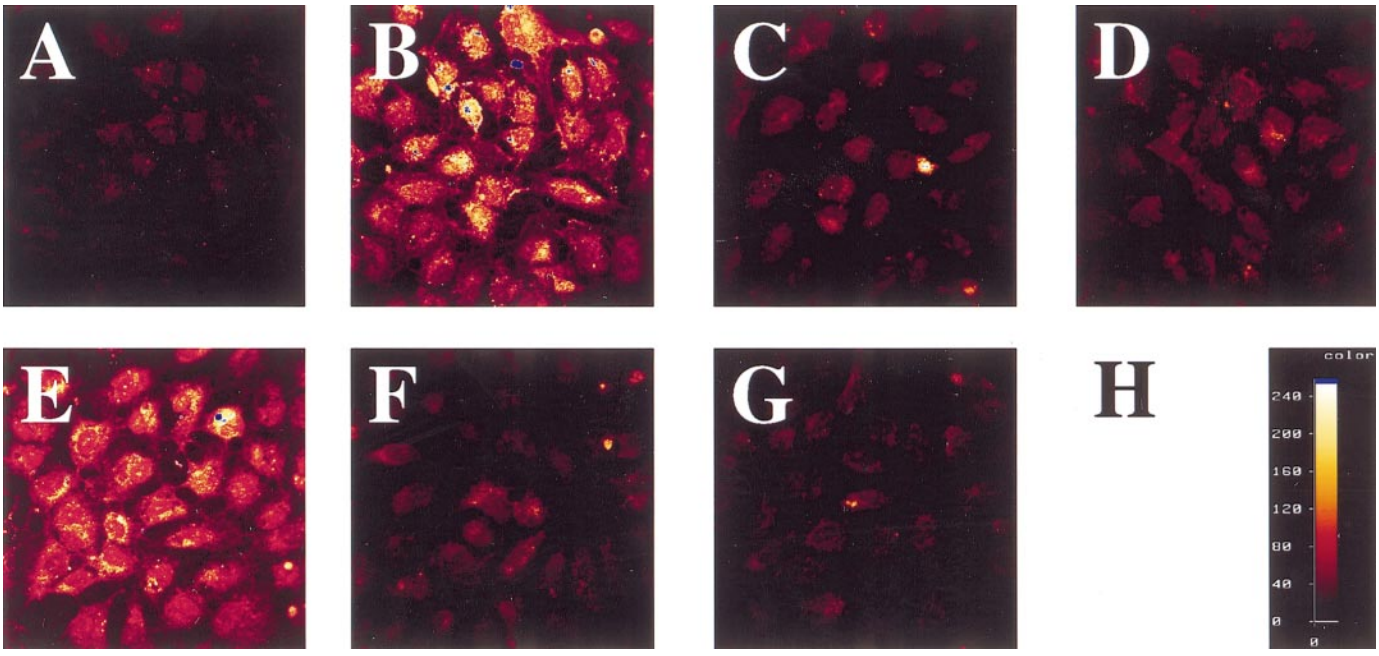

Fig. 1. Confocal laser scanning microscopy (CLSM) analysis of intercellular adhesion molecule-1 (ICAM-1) expression in human umbilical vein endothelial cells (HUVECs) following ischemia/reperfusion injury (I/R) and hyperbaric oxygen (HBO). *A*: control treated cells. *B*: lipopolysaccharide (LPS) treatment at 1 μg/ml for 4 h followed by 20 h of control media. *C*: hypoxia for 4 h followed by normoxia for 20 h. *D*: hypoglycemia for 4 h followed by normoglycemia for 20 h. *E*: hypoxia/hypoglycemia for 4 h followed by normoxia/normoglycemia for 20 h. *F*: hypoxia/hypoglycemia for 4 h followed by HBO at 2.5 atmosphere absolute (ATA) for 1.5 h, then normoxia/normoglycemia for 18.5 h. *G*: HBO at 2.5 ATA for 1.5 h, normoxia for 22.5 h. *H*: color bar intensity wedge corresponding to fluorescence intensity scale. Photomicrographs taken from representative fields.

(Fig. 1*G*). Similar results were obtained using BAECs and an identical immunohistochemical staining protocol (Fig. 2). Analysis of fluorescence intensity staining for ICAM-1 under the various experimental conditions is shown in Table 1.

After exposure to hypoxia/hypoglycemia, treatment of cells with normobaric 100% oxygen or a N_2/O_2 gas mixture equivalent to air at 2.5 ATA did not reduce hypoxia/hypoglycemia-induced ICAM-1 levels (data not shown). Further experiments were performed to establish whether a threshold pressure was required to suppress ICAM-1 induction. After exposure to hypoxia/hypoglycemia, cells were treated with HBO at increasing pressures of 1.5, 2.0, and 2.5 ATA followed by normoxia/normoglycemia. Analysis of ICAM-1 by CLSM in Table 2 shows that a pressure of 2.5 ATA was required for maximal inhibition of hypoxia/hypoglycemia-induced ICAM-1. Increasing pressure showed an incremental suppression of ICAM-1 expression; however, the greatest reduction occurred between 2.0 and 2.5 ATA.

The kinetics of ICAM-1 induction by hypoxia/hypoglycemia and response to HBO treatment were also studied. HUVECs were exposed to hypoxia/hypoglycemia for 4 h with or without treatment with HBO at 2.5 ATA as described in MATERIALS AND METHODS. At increasing intervals following the return to normoxia/normoglycemia, cells were fixed and analyzed by CLSM for ICAM-1 expression. An increase in ICAM-1 expression was first noted 4 h following hypoxia/hypoglycemia, with levels continuing to rise over a 24-h period (Fig. 3). HUVECs treated with HBO showed a blunted rise in ICAM-1 expression, with a small but significant increase noted only at 8 h following hypoxia/hypoglycemia (Fig. 3).

Table 1. *Hypoxia and hypoglycemia induce endothelial cell ICAM-1 protein expression: HBO exposure downregulates hypoxia/hypoglycemia-induced ICAM-1*

Condition	Mean Fluorescence Intensity	
	BAEC	HUVEC
Control	15 ± 8	21 ± 5
LPS	252 ± 8*	226 ± 9*
Hypoxia	17 ± 2	38 ± 2
Hypoglycemia	45 ± 11	29 ± 8
Hypoxia/hypoglycemia	186 ± 3*	203 ± 4*
Hypoxia/hypoglycemia/HBO	15 ± 10	41 ± 1*

Values are means ± SE fluorescence. Bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) were exposed to lipopolysaccharide (LPS), hypoxia, hypoglycemia, and hypoxia/hypoglycemia for 4 h followed by 20 h of normoxia/normoglycemia as described in text. Hyperbaric oxygen (HBO)-treated cells were exposed to hypoxia/hypoglycemia for 4 h followed by 1.5 h of HBO at 2.5 atmosphere absolute (ATA) and then 18.5 h of normoxia/normoglycemia. At 24 h, cell surface intercellular adhesion molecule–1 (ICAM-1) expression was analyzed by confocal laser scanning microscopy (CLSM) as described in text. *$P < 0.05$ for control vs. individual conditions using ANOVA and Fisher's PLSD test with a significance level of 5%.

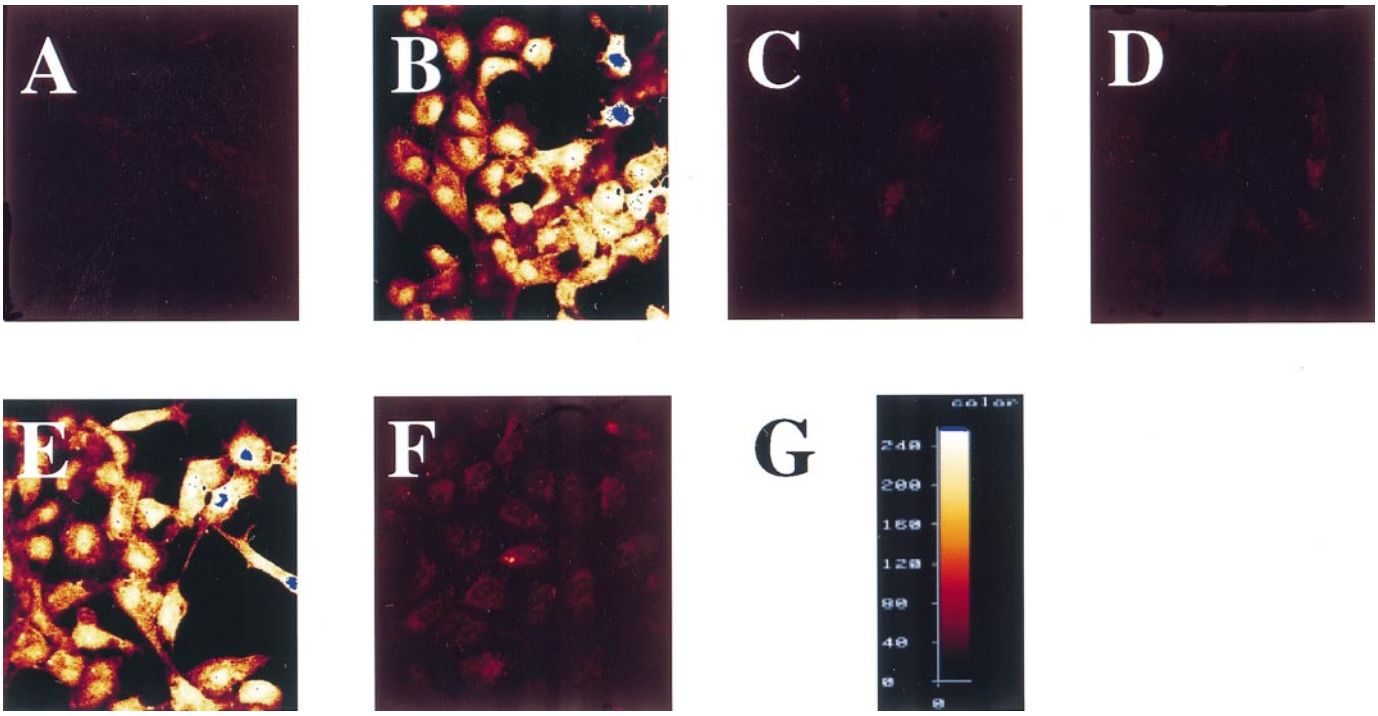

Fig. 2. CLSM analysis of ICAM-1 expression in bovine aortic endothelial cells (BAECs) following I/R and HBO. *A*: control-treated cells. *B*: LPS treatment at 1 μg/ml for 4 h followed by 20 h of control media. *C*: hypoxia for 4 h followed by normoxia for 20 h. *D*: hypoglycemia for 4 h followed by normoglycemia for 20 h. *E*: hypoxia/hypoglycemia for 4 h followed by normoxia/normoglycemia for 20 h. *F*: hypoxia/hypoglycemia for 4 h followed by HBO at 2.5 ATA for 1.5 h, then normoxia/normoglycemia for 18.5 h. *G*: color bar intensity wedge corresponding to fluorescence intensity scale. Photomicrographs taken from representative fields.

Table 2. *Effect of increasing pressures of HBO exposure on hypoxia/hypoglycemia-induced ICAM-1 expression*

Condition	Mean Fluorescence Intensity
Control	28 ± 2
Hypoxia/hypoglycemia	181 ± 4*
Hypoxia/hypoglycemia/HBO 1.5 ATA	164 ± 4*
Hypoxia/hypoglycemia/HBO 2.0 ATA	131 ± 5*
Hypoxia/hypoglycemia/HBO 2.5 ATA	18 ± 2

Values are means ± SE fluorescence. HUVECs were exposed to hypoxia/hypoglycemia for 4 h followed by 20 h of normoxia/normoglycemia as described in text. HBO-treated cells were exposed to hypoxia/hypoglycemia for 4 h followed by 1.5 h of HBO at 1.5, 2.0, or 2.5 ATA and then 18.5 h of normoxia/normoglycemia. At 24 h, cell surface ICAM-1 expression was analyzed by CLSM as described in text. *$P < 0.05$ for control vs. individual conditions using ANOVA and Fisher's PLSD test with a significance level of 5%.

Cell surface ELISA results for ICAM-1 using the hypoxia/hypoglycemia model paralleled those observed with CLSM (Table 3). Cells were subjected to control media, LPS at 1 μg/ml, hypoxia, hypoglycemia, or hypoxia/hypoglycemia for 4 h, followed by 20 h of normoxia/normoglycemia. HBO-treated cells were exposed to 4 h of hypoxia/hypoglycemia for 4 h, transferred to normoglycemic media, and then exposed to HBO at 2.5 ATA for 1.5 h. After HBO exposure, cells were incubated in normoxic/normoglycemic conditions for 18.5 h. Immunoassay of cell surface ICAM-1 demonstrated significant upregulation of ICAM-1 protein in

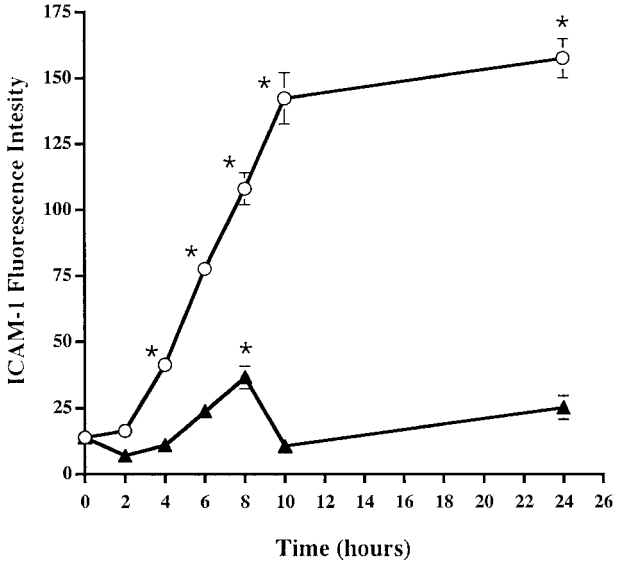

Fig. 3. Effect of HBO on ICAM-1 induction following hypoxia/hypoglycemia. HUVECs were exposed to the following conditions: 4 h of hypoxia/hypoglycemia and then normoxia/normoglycemia for 20 h (○); 4 h of hypoxia/hypoglycemia, then single treatment of HBO at 2.5 ATA for 1.5 h followed by normoxia/normoglycemia for 18.5 h (▲). Cells were analyzed for ICAM-1 protein expression by CLSM as described in text at varied times beginning after exposure to hypoxia/hypoglycemia (time = 0). Fluorescence intensity is expressed as means ± SE. *$P < 0.05$ vs. time = 0 vs. individual time points using one-way ANOVA with Fisher's protected least-significant differences test and a significance level of 5%.

Table 3. *Effect of HBO on hypoxia/hypoglycemia-induced ICAM-1 cell surface protein*

Condition	$OD_{405} \times 1{,}000$	*P*
Control	184 ± 9	ND
LPS (1 μg/ml)	556 ± 16	<0.01
Hypoglycemia	205 ± 17	0.629
Hypoxia	287 ± 23	0.020
Hypoxia/hypoglycemia	479 ± 16	<0.01
Hypoxia/hypoglycemia/HBO	17 ± 2	<0.01

Values are means ± SE optical density $(OD)_{405}$. HUVECs were exposed to LPS, hypoxia, hypoglycemia, and hypoxia/hypoglycemia for 4 h followed by 20 h of normoxia/normoglycemia as described in text. HBO-treated cells were exposed to hypoxia/hypoglycemia for 4 h followed by 1.5 h of HBO at 2.5 ATA and then 18.5 h of normoxia/normoglycemia. At 24 h, cell surface ICAM-1 expression was analyzed by ELISA as described in text. *P* vs. control for individual conditions using ANOVA and Fisher's PLSD test with a significance level of 5%. ND, not done.

the LPS (3-fold, $P < 0.01$) and hypoxia/hypoglycemia groups (2.6-fold, $P < 0.01$). A smaller increase in ICAM-1 was seen following hypoxia (1.5-fold, $P < 0.02$). Hypoglycemia alone did not lead to significant increases in ICAM-1 (1.1-fold, $P = 0.63$). HBO exposure of cells incubated under hypoxic/hypoglycemic conditions led to a 28-fold decrease in ICAM-1 expression vs. cells exposed to hypoxia/hypoglycemia alone ($P < 0.01$). HBO also decreased ICAM-1 cell surface protein expression relative to normoxic/normoglycemic controls by 11-fold ($P < 0.01$).

HBO treatment decreases total protein expression of ICAM-1 in hypoxia/hypoglycemia-stimulated endothelial cells. Western blot analysis of total ICAM-1 protein was performed on both HUVECs and BAECs subjected to similar conditions as described above for CLSM and ELISA analysis. Hypoxia or hypoglycemia were insufficient to induce ICAM-1 protein expression in either HUVECs or BAECs [Fig. 4*A*: HUVECs, compare *lanes 1, 3,* and *4* and corresponding optical density (OD) values 7.3, 4.3, and 4.4 for control, hypoglycemic, and hypoxic conditions, respectively; Fig. 4*B*: compare *lanes 1* and *3* and corresponding OD values 0.0 and 0.2 for control and hypoglycemic conditions, respectively]. In both HUVECs and BAECs, the combination of hypoxic and hypoglycemic conditions vs. media controls induced an ~90–100 kDa protein corresponding to ICAM-1 (Fig. 4*A*, compare *lanes 1* and *5* with corresponding OD values 0.0 and 20.7; Fig. 4*B*, compare *lanes 1* and *4* with corresponding OD values 6.38 and 10.0). The combination of hypoxia/hypoglycemia was similar to LPS (0.5 μg/ml) as an inducer of ICAM-1 in BAECs (Fig. 4*B*, compare *lanes 2* and *4* with corresponding OD values 22.0 and 20.7); however, the LPS-mediated induction of ICAM-1 in HUVECs was ~4.4-fold greater than hypoxia/hypoglycemia (Fig. 4*A*, compare *lanes 2* and *5* with OD values 43.5 and 10.0). The structural protein β-tubulin was used to control for equivalent protein loading of gels (Fig. 4).

HBO exposure decreases adhesion of PMNs to hypoxia/hypoglycemia-stimulated endothelial cell monolayers. We sought to determine whether the observed changes in ICAM-1 protein expression corresponded to

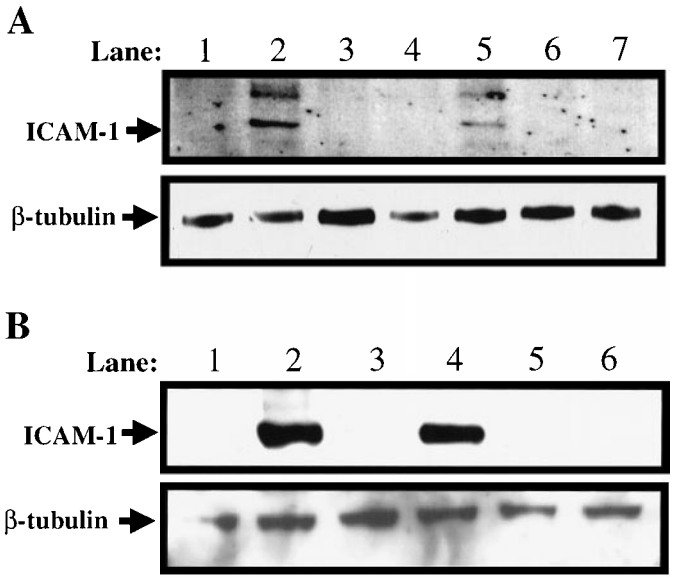

Fig. 4. Western blot analysis of total protein from HUVECs and BAECs following mock I/R injury and the effects of HBO on ICAM-1 expression. *A*: HUVEC *lane 1*, control media 24 h; *lane 2*, LPS (0.5 μg/ml) 4 h followed by 20 h control media; *lane 3*, hypoxia 4 h followed by 20 h normoxia; *lane 4*, hypoglycemia 4 h followed by 20 h normoglycemia; *lane 5*, hypoxia/hypoglycemia 4 h followed by normoxia/normoglycemia 20 h; *lane 6*, hypoxia/hypoglycemia 4 h followed by HBO at 2.5 ATA for 1.5 h, then normoxia/normoglycemia 18.5 h; *lane 7*, HBO at 2.5 ATA for 1.5 h followed by 22.5 h normoxia. *B*: BAEC *lane 1*, control media 24 h; *lane 2*, LPS (0.5 μg/ml) 4 h followed by 20 h control media; *lane 3*, hypoglycemia 4 h followed by 20 h normoglycemia; *lane 4*, hypoxia/hypoglycemia 4 h followed by normoxia/normoglycemia for 20 h; *lane 5*, hypoxia/hypoglycemia 4 h followed by HBO at 2.5 ATA for 1.5 h, then normoxia/normoglycemia 18.5 h; *lane 6*, HBO at 2.5 ATA for 1.5 h, followed by 22.5 h normoxia. Arrows, positions of ICAM-1 and β-tubulin.

a functional difference in static PMN binding to endothelial cells. BAECs were exposed to hypoxia/hypoglycemia followed by normoxia/normoglycemia. One group of cells was treated with HBO as described above. Binding of PMNs was assessed at 24 h relative to the initiation of hypoxia/hypoglycemia (Table 4). PMN binding to hypoxia/hypoglycemia-stimulated BAECs was increased 3.4-fold relative to controls ($P < 0.01$). Treatment of hypoxia/hypoglycemia-stimulated BAECs with HBO decreased PMN binding to control levels (control vs. hypoxia/hypoglycemia/HBO, $P = 0.70$).

Table 4. *Effect of HBO on hypoxia/hypoglycemia-induced PMN/endothelial cell adhesion*

Condition	$OD_{405} \times 1{,}000$	*P*
Control	171 ± 17	ND
Hypoxia/hypoglycemia	580 ± 73	<0.01
Hypoxia/hypoglycemia/HBO	144 ± 5	0.667

Values are means ± SE OD_{405}. BAECs were exposed to hypoxia/hypoglycemia for 4 h followed by 20 h of normoxia/normoglycemia as described in text. HBO-treated cells were exposed to hypoxia/hypoglycemia for 4 h followed by 1.5 h of HBO at 2.5 ATA and then 18.5 h of normoxia/normoglycemia. At 24 h, peripheral blood polymorphonuclear leukocytes (PMNs) were used in the PMN/endothelial cell adhesion assay as described in the MATERIALS AND METHODS. *P* vs. control for individual conditions using ANOVA and Fisher's PLSD test with a significance level of 5%.

HBO exposure induces synthesis of eNOS protein. Previous studies have suggested that hyperoxia leads to increased production of NO and eNOS protein (1, 32). We wished to determine whether HBO exposure influenced the expression of eNOS. HUVECs were subjected to 90 min of HBO at 2.5 ATA, and eNOS protein expression was analyzed at increasing time intervals using fluorescence intensity staining as determined by CLSM. A single HBO exposure led to an increase in eNOS protein production (Fig. 5). iNOS was not detectable in HUVECs following HBO exposure (Fig. 5). LPS-mediated induction of iNOS, but not eNOS, was observed in RAW 264.7 macrophages at 24 h in control studies (Fig. 5). Figure 6 demonstrates the induction of eNOS over 24 h. eNOS expression was significantly increased 2 h following the initiation of HBO and continued to rise, with the greatest increase in expression occurring between 6–8 h. Peak expression was noted at 8 h, and no decrease in eNOS protein levels was noted at 24 h.

eNOS total protein production was similarly upregulated following HBO exposure as determined by Western blot analysis. eNOS protein was induced by exposure of HUVECs to HBO (Fig. 7, compare *lanes 1*, *5*, and *6*). eNOS protein levels were increased 5.2-fold in the hypoxia/hypoglycemia/HBO-treated cells vs. controls (Fig. 7, compare *lanes 1* and *5* with respective OD values 1.27 vs. 6.2). HUVECs treated with HBO alone produced a 3.8-fold greater amount of eNOS protein at 24 h vs. the hypoxia/hypoglycemia/HBO-treated cells (Fig. 7, compare *lanes 5* and *6* with respective OD values 23.5 vs. 6.2). Hypoxia-, hypoglycemia-, or hypoxia/hypoglycemia-treated cells did not express detectable levels of eNOS protein by Western blot analysis (Fig. 7, lanes *2*, *3*, and *4* with respective OD values 2.2, 2.3, and 2.1). However, low levels of eNOS were detectable in HUVECs by CLSM.

Inhibition of eNOS with L-NAME attenuates HBO-mediated downregulation of ICAM-1. Previous studies have shown that organic NO donors can block the cytokine-mediated induction of ICAM-1 (10). Therefore, we sought to determine whether the induction of eNOS protein was related to the observed downregulation of ICAM-1. HUVECs were subjected to hypoxia/hypoglycemia as described above, and L-NAME was added to the culture media on return of the cells to normoxic/normoglycemic conditions. ICAM-1 expression was analyzed using CLSM as described in MATERIALS AND METHODS. L-NAME had minimal effect on ICAM-1 protein expression in the normoxia/normoglycemia control group, with only a significant 1.96-fold increase noted at 100 μM L-NAME ($P < 0.01$; Fig. 8). This finding was in keeping with the low levels of eNOS protein observed in the control cells. Hypoxia/hypoglycemia-treated cells incubated with L-NAME at a concentration of 25 μM did not increase ICAM-1 levels significantly above those from hypoxia/hypoglycemia-only treated cells ($P = 0.93$). A small response was observed in hypoxia/hypoglycemia-treated cells incubated with L-NAME at concentrations of 50 and 100 μM, demonstrating a 1.2- and 1.4-fold induction of ICAM-1 vs.

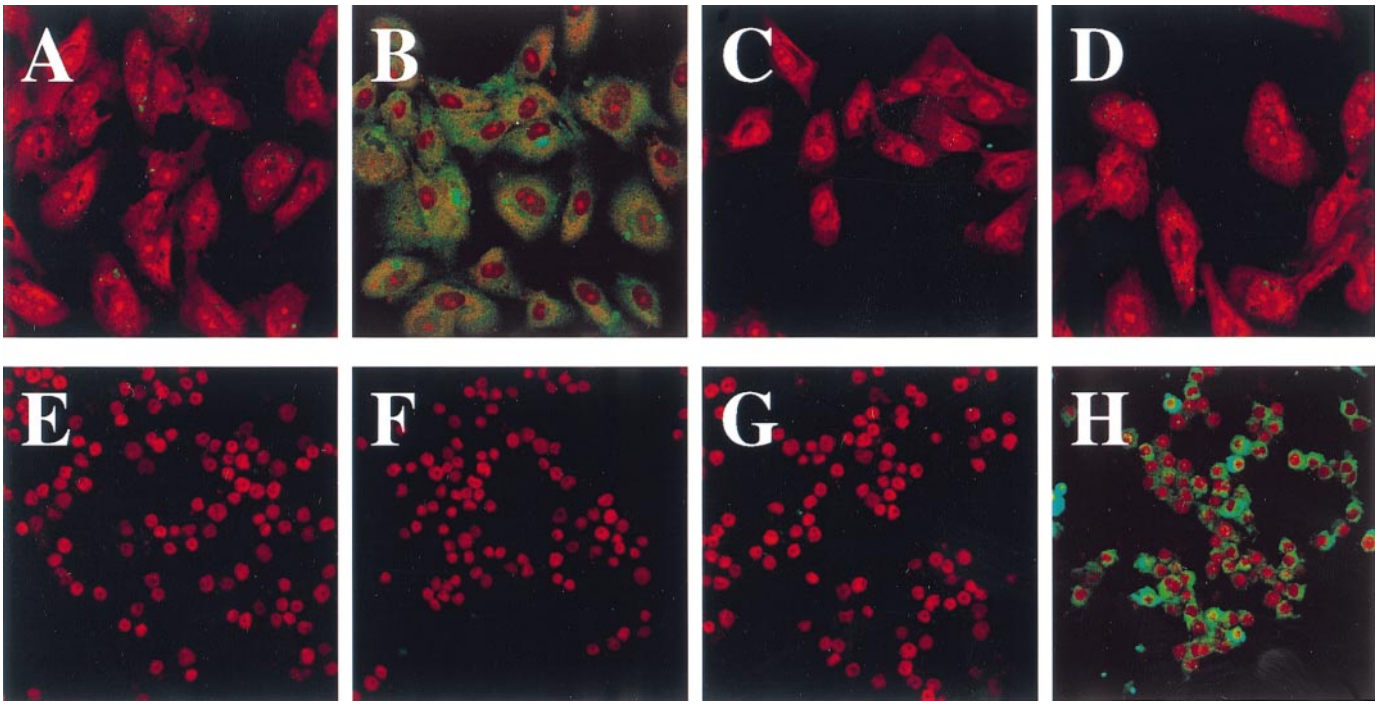

Fig. 5. CLSM analysis of endothelial cell nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) expression. Cells were stimulated as follows and analyzed by CLSM as described in text (red channel indicates propidium iodide staining of nucleic acid; green channel indicates specific antibody staining). *A*: eNOS expression in HUVEC controls. *B*: eNOS expression in HUVECs exposed to hypoxia/hypoglycemia for 4 h followed by HBO treatment at 2.5 ATA for 1.5 h, followed by normoxia for 18.5 h. *C*: iNOS expression in HUVEC controls. *D*: iNOS expression in HUVECs exposed to hypoxia/hypoglycemia for 4 h followed by HBO treatment at 2.5 ATA for 1.5 h, followed by normoxia for 18.5 h. *E*: eNOS expression in control RAW 264.7 cells. *F*: eNOS expression in RAW 264.7 cells stimulated with LPS at 500 ng/ml for 4 h, followed by 20 h of control media. *G*: iNOS expression in control RAW 264.7 cells. *H*: iNOS expression in RAW 264.7 cells stimulated with LPS at 500 ng/ml for 4 h, followed by 20 h of control media.

hypoxia/hypoglycemia-treated cells not receiving L-NAME ($P < 0.01$). The relative response of control and hypoxia/hypoglycemia-treated cells to L-NAME was similar with respect to increases in ICAM-1 levels. The greatest effect of eNOS inhibition was observed in the hypoxia/hypoglycemia/HBO-treated cells. Addition of 25 μM L-NAME led to a relative threefold increase in ICAM-1 levels in hypoxia/hypoglycemia/HBO-treated cells ($P < 0.01$). Hypoxia/hypoglycemia-stimulated HUVECs treated with HBO and L-NAME demonstrated a dose-dependent increase in ICAM-1 protein expression of 3.0-, 4.4-, and 4.7-fold at concentrations of 25, 50, and 100 μM, respectively ($P < 0.01$). HBO-only treated cells containing the highest levels of eNOS demonstrated only a modest increase in ICAM-1 with L-NAME inhibition (1.6-fold at 100 μM, $P < 0.01$). Taken together, these findings suggest that the HBO downregulation of hypoxia/hypoglycemia-induced

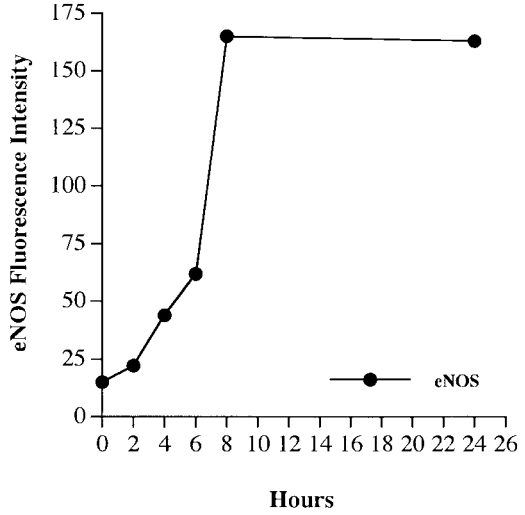

Fig. 6. Induction of eNOS protein as determined by CLSM analysis. HUVECs were exposed to a single treatment of HBO at 2.5 ATA for 1.5 h, and eNOS expression was analyzed at the indicated time points. Fluorescence intensity is determined as described in MATERIALS AND METHODS. Time = 0 indicates initiation of HBO treatment.

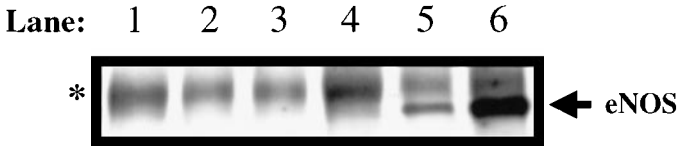

Fig. 7. eNOS and iNOS expression in HUVECs. *Lane 1*, control media 24 h; *lane 2*, hypoxia 4 h, followed by 20 h normoxia; *lane 3*, hypoglycemia 4 h, followed by 20 h normoglycemia; *lane 4*, hypoxia/hypoglycemia 4 h, followed by normoxia/normoglycemia 20 h; *lane 5*, hypoxia/hypoglycemia 4 h, followed by HBO at 2.5 ATA for 1.5 h, then normoxia/normoglycemia 18.5 h; *lane 6*, HBO at 2.5 ATA for 1.5 h, followed by 22.5 h normoxia. Arrow, position of eNOS. *Position of nonspecific protein.

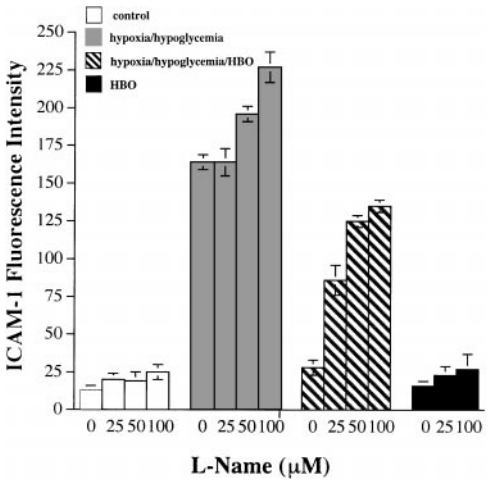

Fig. 8. nitro-L-arginine methyl ester (L-NAME) attenuates the HBO-mediated downregulation of ICAM-1 expression in HUVECs. HUVECs were exposed to normoxia/normoglycemia control media (open bars); hypoxia/hypoglycemia for 4 h followed by normoxia/normoglycemia for 20 h (shaded bars); hypoxia/hypoglycemia for 4 h followed by HBO at 2.5 ATA for 1.5 h, then 18.5 h normoxia/normoglycemia (cross-hatched bars); or HBO at 2.5 ATA for 1.5 h followed by normoxia for 22.5 h (solid bars). L-NAME was added at the initiation of normoxic/normoglycemic conditions and following HBO treatment. ICAM-1 protein expression was analyzed by CLSM as described in MATERIALS AND METHODS.

ICAM-1 may be mediated, at least in part, through increased expression of eNOS.

DISCUSSION

The molecular mechanisms of HBO in treating I/R injury are poorly understood. We have established an in vitro model of I/R injury to study the effects of HBO on endothelial cell function. Our model of endothelial cell I/R required both hypoxia and hypoglycemia for induction of ICAM-1 protein production. The combination of hypoxia/hypoglycemia has been used previously for study of neuronal cell responses to ischemia in vitro (28). We are not aware of any previous reports describing the specific role of hypoxia and hypoglycemia as an inducer of ICAM-1 protein production. Previous studies utilizing BAECs and HUVECs have suggested that hypoxia alone, or in conjunction with a reoxygenation period, were insufficient to induce ICAM-1 (29, 49, 53). Similarly, we found that hypoxia or hypoxia/reoxygenation were insufficient to stimulate the induction of ICAM-1 protein. Hypoxia has been shown to enhance the production of ICAM-1 in combination with LPS (54). In these studies, hypoxia promoted increased intracellular acidosis, resulting in an increase of ICAM-1 expression via a proteasome-dependent pathway (54). It is possible that the combination of hypoxia and hypoglycemia exacerbates the hypoxia-induced intracellular acidosis or ATP depletion, leading to induction of ICAM-1 (25).

The ability of HBO to suppress the production of ICAM-1 in our model may help explain the beneficial mechanism of HBO in treating I/R injury. The observation that HBO decreases adhesion of PMNs to the endothelium and also overall PMN content within previously ischemic tissue has been well described (50). Investigation of the ICAM-1 ligand CD11/CD18 on PMNs suggested that there was no long-term alteration of inducible CD11/CD18 function following HBO exposure (44, 45). One study suggested a functional inhibition of PMN β_2-integrins using HBO, although this study did not determine the specific interaction of CD11/CD18 and ICAM-1 (46). Our findings of a reduction in non-HBO-treated PMN adhesion to HBO-treated endothelial cells suggests that the underlying mechanism of HBO in treating I/R injury in vivo may be mediated by effects on both the endothelium and PMNs. It is possible that other endothelial cellular adhesion molecules are similarly downregulated by HBO. Finally, the influence of HBO appears to modulate the expression of endothelial cell proteins specifically; as ICAM-1 production is decreased, eNOS is increased, and β-tubulin levels remain constant.

Our finding that HBO was able to reverse the expression of ICAM-1 in vitro differs from expected results suggested by previous studies of hyperoxia and ICAM-1 expression. Others have shown that prolonged hyperoxia is toxic to endothelial cells and lung tissue, inducing the synthesis of ICAM-1 under both normobaric and prolonged hyperbaric conditions (3, 36, 43). We similarly found that normobaric O_2 exposure following experimental I/R did not decrease ICAM-1 production in our model. It is possible that the difference in the ICAM-1 response to hyperoxia is due to the state of cellular metabolism (I/R vs. resting) and the P_{O_2} level and duration of exposure. These different conditions may result in varied quantitative and qualitative production of ROS. Few studies have assessed the amount of ROS-induced damage following HBO treatment in the setting of I/R. One study demonstrated no significant long-term increase in cerebral lipid peroxidation following transient global ischemia with subsequent HBO treatment (24). Others have observed a transient, nonsustained increase in lipid peroxidation following HBO at extreme pressures of 5 ATA (26). Overall, however, HBO reduces tissue damage using in vivo I/R injury models, an outcome less likely predicted in a setting of oxidative stress and free radical damage (16, 41, 42, 50).

Media P_{O_2} was not measured directly in our model; however, an extensive previous study has documented the P_{O_2} dissolved in solution in vitro under similar hyperbaric conditions compared with the P_{O_2} of human arterial blood from subjects undergoing HBO exposure (47). In these previous experiments, the P_{O_2} of saline reached 1,509 and 1,842 mmHg at 2.0 and 2.5 ATA, respectively, after 20 min, whereas the approximate equivalent P_{O_2} of human arterial blood required exposure to 2.2 and 2.9 ATA, respectively (47). Our results suggest that the threshold dose for inhibition of ICAM-1 expression in vitro exists between the P_{O_2} generated in solution between 2.0 and 2.5 ATA. Because current human treatment protocols include HBO exposure at up to 3.0 ATA, the P_{O_2} required to achieve a reduction in ICAM-1 production observed in vitro should be obtainable in the clinical setting, especially in the microcircu-

lation of the myocardium. Although our findings suggest that a threshold dose for inhibition of ICAM-1 expression should exist in vivo, it is not possible to extrapolate our findings directly into clinical treatment protocols without appropriate further in vivo studies.

Data demonstrating eNOS induction with HBO exposure are consistent with the literature. Other reports have shown that increases in oxygen tension at 1 ATA lead to an induction of eNOS mRNA and protein production (1, 27). Increases in NO production were noted in bovine cerebellum following HBO exposure by direct measurement; however, direct analyses of NOS protein levels or subtype were not performed (32). We have demonstrated that eNOS protein levels are upregulated following HBO. Our findings of low constitutive eNOS levels have been similarly described (21). The sustained increase in eNOS following a single HBO exposure may explain the previous observation that HBO treatment results in greater vessel diameter of the microvasculature following I/R injury (50). Previous study of porcine coronary resistance arterioles showed a similar phenomenon of hyperoxia-induced NO production with vasodilation (15). The sustained HBO-mediated increase in eNOS protein levels may also help explain the protective effect of HBO treatment given before I/R injury in a rodent liver model (5). Although we have not directly measured NO production, it is likely that our results follow from an increase in NO. This is supported by experiments demonstrating that L-NAME competitive inhibition of NOS resulted in enhanced production of ICAM-1. The lack of iNOS expression noted in our model system by CLSM suggests that eNOS is primarily responsible for the observed results; however, we have not characterized the enzymatic requirements of NOS for calcium dependence. The inability of L-NAME to completely reverse the HBO-mediated suppression of ICAM-1 allows for the existence of other undefined pathways for HBO regulation of ICAM-1 expression.

The regulation of cellular adhesion molecules by NO has been well described (10, 20, 22). Recent studies suggest that the NO-mediated downregulation of ICAM-1 may occur via a reduction in activated NF-κB, which may involve increased production of IκB-α (21, 31, 40). One hypothesis to explain our data is that HBO reduces the activation of transcription factors such as NF-κB involved in the transcriptional regulation of ICAM-1 through a NO-mediated mechanism (18). NO may combine with other ROS formed during states of oxidative stress to prevent the ROS from initiating other stress responses. Other studies have shown that NF-κB may be activated in endothelial cells following hypoxia/reoxygenation and also under states of oxidative stress induced by H_2O_2 or chronic O_2 exposure at 1 ATA (7, 31, 33–35). If the effect of HBO is mediated through NO and NF-κB, then other similarly regulated adhesion molecules may be downregulated following HBO exposure.

In summary, we have shown that HBO effectively downregulates ICAM-1 expression in an in vitro model of endothelial cell I/R injury. The corresponding increase in eNOS and inhibitor studies suggest that the mechanism of downregulation may be mediated in part by NO. These findings further define the mechanisms behind the observed paradoxical benefit of HBO-induced hyperoxia in the setting of I/R injury. Verification of these findings in vivo would further support the clinical use of HBO as a therapeutic intervention in I/R disease states.

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